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A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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COMPARATIVE GONADOTROPIN ASSAYS AND USE OF CALCIUM PHOSPHATE AS AN ADSORBENT IN CONCENTRATION OF FOLLICLE-STIMULATING ACTIVITY FROM PITUITARY GLAND[1]

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VERY SOON after the discovery of the gonadotropic activity of the pituitary with implants and extracts by Smith (1) and Zondek and Aschheim (2), it became obvious that the biological response was a complex one (3). At present, the most commonly accepted view is that two specific biological responses are involved, and that they are due to two specific substances (4), the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). The former causes follicular growth and maturation in the ovary, and spermatogenesis in the testis. The latter causes luteinization of the mature follicles in the ovary, and growth of the interstitial cells in the testis. Although the Evans (5) and the van Dyke (6) groups have reported the isolation of the luteinizing hormone in pure form, devoid of follicle-stimulating activity, nevertheless neither factor has been obtained in crystalline form.

The problem of separating and purifying the FSH and the LH factors is especially involved because of the difficulty of assaying each factor accurately when in a supposedly pure state and when mixed with other factors. Theoretically, hypophysectomized animals should be used in all of these assays and in particular in the testing of the final products. However, because of the cost and difficulty of preparing and maintaining large numbers of hypophysectomized animals, it was deemed expedient, in the case of preliminary fractionations, to use immature normal animals. Consequently, a number of comparative assays on glandular preparations were made with immature normal rats and mice, and in some cases with hypophysectomized rats.

MATERIALS ASSAYED

The pituitary products compared in these studies were acetone desiccated sheep pituitary powder and two concentrates therefrom, '*powder A*' and '*powder EB*'.

The acetone-desiccated sheep pituitary powder was obtained by repeatedly dehydrating finely ground, fresh, whole pituitary glands from sheep with acetone and finally removing the acetone from the tissue residue by means of a fan. The dried material was pulverized to a fine powder in a ball-mill or in a mortar.

Powder A (7) was prepared by adding 4 volumes of ethanol to a 0.02N ammonium-hydroxide extract of desiccated sheep pituitary powder. The extract was adjusted to pH 5.0 to 5.4 before the addition of the ethanol.

Powder EB was prepared from *powder A* by adsorption on tribasic calcium phosphate and elution with dilute alkali. The details of this method are reported later in this paper.

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METHODS OF ASSAY

Desiccated sheep pituitary powder and *powder A* were injected subcutaneously as suspensions and *powder EB* as a clear solution in physiological salt solution.

When immature normal female rats were used, the injections were given twice daily for 3 days. On the morning of the 4th day, the ovaries and uteri were carefully dissected, blotted with paper toweling, and weighed.

When immature normal male rats were used, the injections were given twice daily for 5 days. On the morning of the 6th day, the testes, seminal vesicles, anterior lobe of the prostate gland, and the adrenal glands were similarly removed and weighed.

The mouse uterine assay method was the same as that employed by J. S. Evans et al. (8) and similar to that of Hamburger and Pedersen-Bjergaard (9). Immature normal female mice were injected twice daily for 3 days. Thirty-six hours after the last injection, the uteri were removed, blotted with paper toweling, and weighed.

The hypophysectomized, immature male or female rats were injected twice daily for 4 days, and the autopsy was conducted on the 5th day. The injections were begun on the second day after the hypophysectomy. This period of time was not chosen arbitrarily, but was found by preliminary experiments to give more consistent results than if a longer rest period after operation were allowed. At autopsy, the top of the cranium was cut away, the brain carefully removed, and an examination made to ascertain whether the hypophysectomy was complete. Serial sections were not made. If the visual examination revealed an incomplete hypophysectomy, the organ weights of that animal were discarded.

In one experiment, *powder A* was assayed by the Marrian and Parkes method (10) in vitamin B_1-deficient young, mature, female rats. Fourteen young rats, 67 days old, were placed on a vitamin B_1-deficient diet consisting of 73 per cent starch, 20 per cent casein, 5 per cent inorganic salt mixture, and 2 per cent cod-liver oil. Within 4 weeks all of the rats developed a state of continuous anestrus. *Powder A* was then injected into these rats once daily for 3 days in an effort to produce a vaginal smear typical of estrus.

All of the rats were raised in our own colony. The mice were purchased from the Maple Grove Rabbitry of Springfield, Missouri.

The results of the assays are shown in tables 1 to 3 and figures 1 to 4. The organ weights are, in each case, average values compiled from one or more individual assays, each based on 5 or more animals.

DISCUSSION OF ASSAY RESULTS

The results of the vitamin B_1-deficiency experiment were as follows. The control rats remained in anestrus; the 3 rats injected with a total dose of 0.01 mg. of *powder A* also remained in anestrus. Two of 3 rats which received 0.1 mg. of *powder A* came into estrus within 4 days, and the 3 rats which received a total dose of 1.0 mg. gave vaginal smears typical of estrus within 2 days of the final injection. If no further injections were administered, the animals again lapsed into anestrus. All of the rats lost weight during the course of the experiment. When these deficient rats were returned to a complete diet, they gained weight rapidly, and when their body weights reached approximately the normal value for their age, the normal estrous cycle reappeared in every case.

It is evident that although the results of the vitamin B_1-deficiency experiment are in the proper order and confirm those of Marrian and Parkes (10), nevertheless such a method would be far too laborious and insensitive to be considered as a general assay method.

It is apparent from the results of the assays made in normal immature female rats (fig. 1), that there is a marked difference in the type of ovarian and uterine responses elicited by injections of *powder A* as compared with desiccated sheep pituitary powder. With *powder A* there occurred a sharp initial rise in the uterine response which tapered off in the vicinity of 50 to 70 mg. of uterine weight. The ovaries showed a gradual, almost linear, response until the weight reached 40 mg. whereupon the response remained on a plateau. On the other hand, the desiccated sheep pituitary powder (DSP) effected a prompt rise in uterine response up to a value of about 90 mg.

TABLE 1. EFFECT OF VARYING DOSES OF *powder A*, *powder EB*, AND DESICCATED SHEEP PITUITARY POWDER UPON THE OVARIAN, UTERINE, AND ADRENAL WEIGHTS OF HYPOPHYSECTOMIZED IMMATURE RATS[1]

Number of Rats Used	Total Dose Injected, mg.	Final Body Weight, gm.	Ovarian Weight, mg.	Uterine Weight, mg.	Adrenal Weight, mg.
		Powder A			
35	0.00	49	7.6	13.8	10.5
4	0.25	42	7.0	15.7	9.1
12	0.50	49	11.9	17.3	9.2
13	1.0	56	19.0	60.6	10.4
4	2.0	56	22.7	58.2	12.3
8	5.0	47	33.3	59.6	9.4
		Powder EB			
6	0.025	43	8.9	24.8	9.2
14	0.05	50	19.7	86.9	9.4
8	0.50	45	53.8	90.5	10.2
		Desiccated Sheep Pituitary Powder			
9	5.0	55	11.4	23.1	11.1
15	10.0	53	19.1	48.4	13.4

[1] Normal female rats, 34 days of age and weighing an average of 53 gm., were found to have an ovarian weight of 13.1 mg., a uterine weight of 20.5 mg., and an adrenal weight of 17.0 mg.

where a plateau resulted. The ovarian weight response was more gradual, but steadily upwards, so that even with the highest dose of desiccated sheep pituitary powder, the ovarian weight, which had already passed the 100-mg. level, was still ascending.

These results indicate that the desiccated sheep pituitary (DSP) powder may have contained some factor which was not present in *powder A* or that the ratio of luteinizing to follicle-stimulating hormone in the two powders was different. The latter explanation is more probable because the assays for the luteinizing activity in hypophysectomized male rats showed that *powder A* consists mainly of follicle-stimulating hormone.

In normal immature male rats (fig. 2), desiccated sheep pituitary powder (DSP), and *powder A*, respectively, produced very similar responses in the testes, but again some differences in the responses of the secondary sex organs (fig. 3). With *powder A* the response of the testes rose sharply with increased dosage and then maintained a plateau effect. The prostate weight response also rose sharply and then showed an irregular plateau effect. The weight of the seminal vesicles increased 50 per cent at once, but then remained at that level even when ten times the minimum dose necessary to effect the 50 per cent increase was administered. With desiccated sheep pituitary powder (DSP), the response of the testes was similar to that effected by

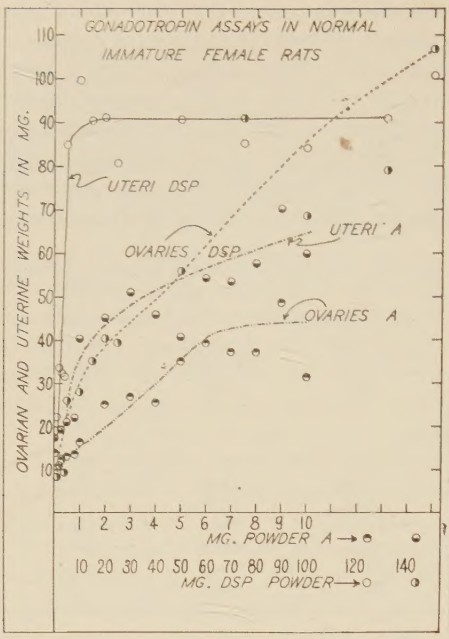


Fig. 1

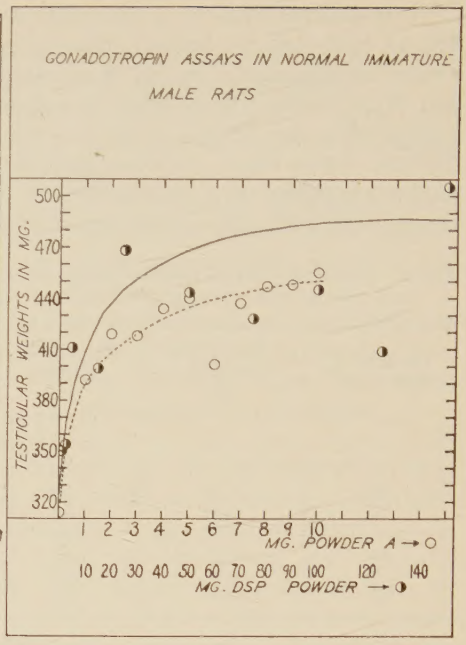


Fig. 2

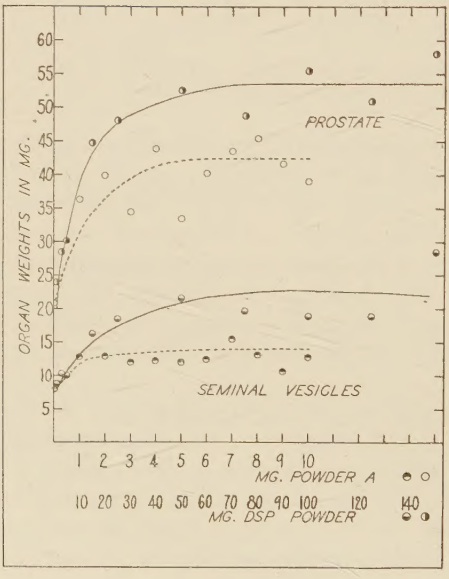


Fig. 3. Gonadotropin assays in normal immature male rats.

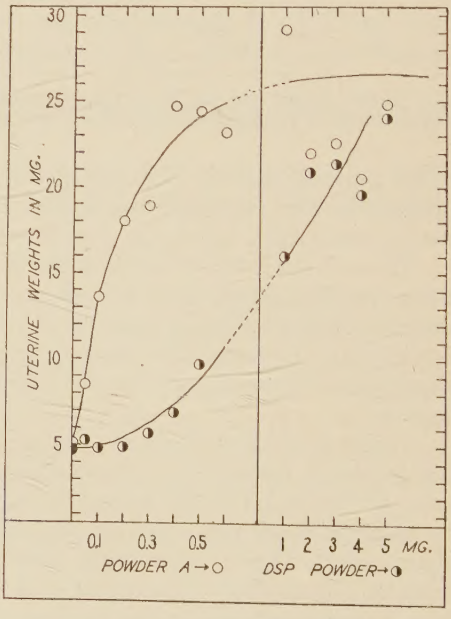


Fig. 4. Gonadotropin assays in normal immature female mice.

powder A. However, there were marked increases in the responses of the prostate and seminal vesicles as compared with the responses obtained with *powder A*. This increase in the weight of the seminal vesicles of normal immature male rats has been used by Fevold et al. (11) to test for luteinizing hormone.

These results again indicate that *powder A* is low in luteinizing activity. In all of our studies with normal immature rats, the adrenal weights remained at control values regardless of the dose of *powder A* or desiccated sheep pituitary powder. These results confirm the view that crude adrenotropic preparations cannot be assayed in normal immature rats.

The mouse uterine assay method yielded very consistent results (fig. 4) and proved

TABLE 2. EFFECT OF VARYING DOSES OF *powder A*, DESICCATED SHEEP PITUITARY POWDER, AND *powder EB* UPON WEIGHTS OF TESTES, SEMINAL VESICLES, ANTERIOR LOBE OF PROSTATE GLAND, AND ADRENAL GLANDS OF HYPOPHYSECTOMIZED IMMATURE RATS[1]

Total Dose Injected, mg.	Final Body Weight, gm.	Testes mg.	Seminal Vesicles, mg.	Prostate Gland, mg.	Adrenal Glands, mg.
		Powder A			
0.0	47 (30)[2]	157	6.3	7.4	9.5
1.0	41 (5)	274	9.9	20.7	7.2
5.0	45 (5)	299	6.7	17.2	10.2
		Powder EB			
0.05	49 (7)	355	7.1	12.3	8.4
0.10	42 (5)	334	6.1	18.7	8.4
0.50	41 (3)	273	5.8	16.6	8.9
		Desiccated Sheep Pituitary Powder			
5.0	46 (5)	225	8.6	18.0	10.9
10.0	47 (7)	252	11.4	32.9	10.5

[1] Normal male rats, 34 days of age and weighing an average of 47 gm., were found to have a testicular weight of 268 mg., a seminal vesicle weight of 7.0 mg., a prostate weight of 19.1 mg., and an adrenal weight of 17.3 mg.

[2] The figures in parentheses designate the number of animals used.

to be the most sensitive method for measuring follicle-stimulating activity. This method of assay indirectly measures the effect of the follicle-stimulating principle through the secretion of estrogens by the follicles. The results of the assays showed desiccated sheep pituitary powder (DSP) to be about one-tenth as active, weight for weight, as *powder A*. The responses obtained varied slightly with the temperature of the environment, for it was found that the uterine response was about 20 per cent higher during the hot months than during the cold months.

Powder EB was found to be very active by the mouse uterine method, as little as 0.006 mg. causing a 50 to 100 per cent increase in the uterine weight.

These results confirm the earlier reports (8) that the immature mouse uterine weight change is a sensitive assay method for the follicle-stimulating principle. The data show that it is 5 to 10 times as sensitive as the method involving hypophysectomized immature female rats and 20 times as sensitive as the method involving normal immature female rats.

The results of the assays on hypophysectomized rats (table 1) are more significant than the results of assays in which animals with intact pituitary glands are employed,

because the intact pituitary gland of the animal may seriously modify the character of the response. The assays on hypophysectomized female rats indicate that the follicle-stimulating activity in *powder EB* is about 20 times as concentrated as in *powder A* and about 200 times as concentrated as in desiccated sheep pituitary powder.

The hypophysectomized male rats were used to measure the luteinizing activity in terms of the increase in the weight of the prostate gland (table 2). It must however be pointed out that the results on hypophysectomized male rats were based upon fewer animals than the results on hypophysectomized female rats. The results appear

TABLE 3. COMPARISON OF RESULTS OBTAINED FROM ASSAYS ON DESICCATED SHEEP PITUITARY POWDER *powder A*, AND *powder EB*

Material Assayed	FSH Assays	LH Assays
	A) In hypophysectomized female rats	In hypophysectomized male rats
	B) In normal immature female mice	
Acetone-desiccated sheep pituitary powder	A) >5.0 mg.<10.0 mg.=1 R.U.	5.0 mg.≧1 R.U.; however, a 10.0 mg. dose effected an 80% weight increase in seminal vesicles and a 300% weight increase in the prostate
	B) 0.5 mg.=1 M.U.	
Powder A (5% by weight yield from desiccated sheep pituitary powder)	A) >0.5 mg.<1.0 mg.=1 R.U.	1.0 mg.≧1 R.U., a 5.0-mg. dose caused no further increase in prostatic weight; seminal vesicles were in neither case any larger than those of the controls
	B) 0.05 mg.=1 M.U.	
Powder EB (1.5–2.0% by weight yield from *powder A*)	A) >0.025 mg.<0.05 mg.=1 R.U.	0.1 mg.≧1 R.U.; a 0.5-mg. dose effected no further increase in prostatic weight; seminal vesicles were in neither case any larger than the controls
	B) 0.006 mg.=1 M.U.	

100% increase in rat ovarian weight=1 R.U. of FSH.
100% increase in rat prostatic weight=1 R.U. of LH.
50–100% increase in mouse uterine weight=1 M.U. of FSH.

to be clear-cut with the desiccated sheep pituitary powder, for the responses parallel the doses. However, with *powder A* and especially with *powder EB*, the results of the assays are questionable. Although a definite prostate response was elicited in both cases, nevertheless, when 5 times this minimal effective dose was administered, there was no better prostate response, but instead a slightly lower one. Furthermore, although the seminal vesicles increased in weight with desiccated sheep pituitary powder, especially at higher doses, they increased only slightly with *powder A* and not at all when *powder EB* was administered. That *powder EB* has little or no luteinizing activity was substantiated by the histological sections of ovaries from hypophysectomized rats to which the highest doses of *powder EB* had been administered (fig. 5). Microscopic examination of these sections showed numerous follicles and almost no corpora lutea.

Desiccated sheep pituitary powder was the only preparation which caused a weight increase in the adrenal glands of hypophysectomized male and female rats. This indicates that neither *powder A* nor *powder EB* contains any appreciable adrenotropic activity. Furthermore, a few isolated assays in one-day-old cockerels showed that desiccated sheep pituitary powder contains a small, but definite, amount of thyrotropic activity, but that *powder A* contains very little and *powder EB* no thyrotropic activity.

The relative activities of desiccated sheep pituitary powder, *powder A* and *powder EB*, as interpreted from our data, are presented in table 3. Since standard definitions of units for the follicle-stimulating and the luteinizing hormones have not been adopted, we have arbitrarily assigned the units indicated. From the data presented in table 3, it is apparent that on the basis of the mouse assays, *powder EB* is about 9 times as potent as *powder A* and about 90 times as potent as desiccated sheep pituitary powder with respect to follicle-stimulating activity. On the basis of the assays in hypophysectomized female rats, *powder EB* is 20 times as active as *powder A* and 200 times as active as desiccated sheep pituitary powder. The results obtained on the hypophysectomized male rat, as stated previously, are of questionable value except in the case of desiccated sheep pituitary powder.

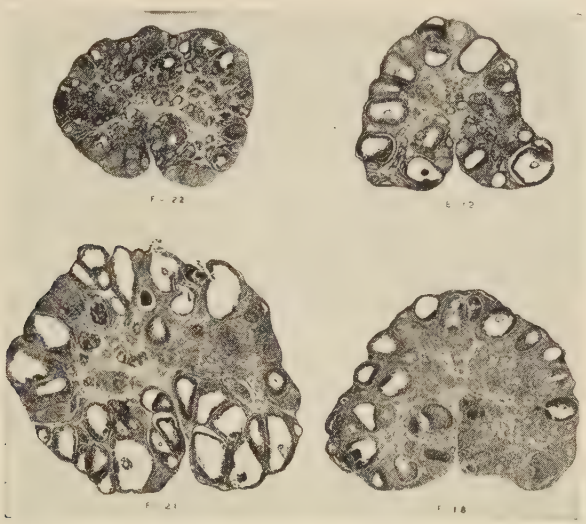


Fig. 5. OVARIES FROM HYPOPHYSECTOMIZED RATS TREATED WITH PITUITARY PREPARATIONS. *Section E-12* shows the effect from 0.05 mg. of *powder EB; section F-21*, 0.50 mg. of *powder EB; section F-18*, 10.0 mg. of desiccated sheep pituitary powder; *section F-22*, control.

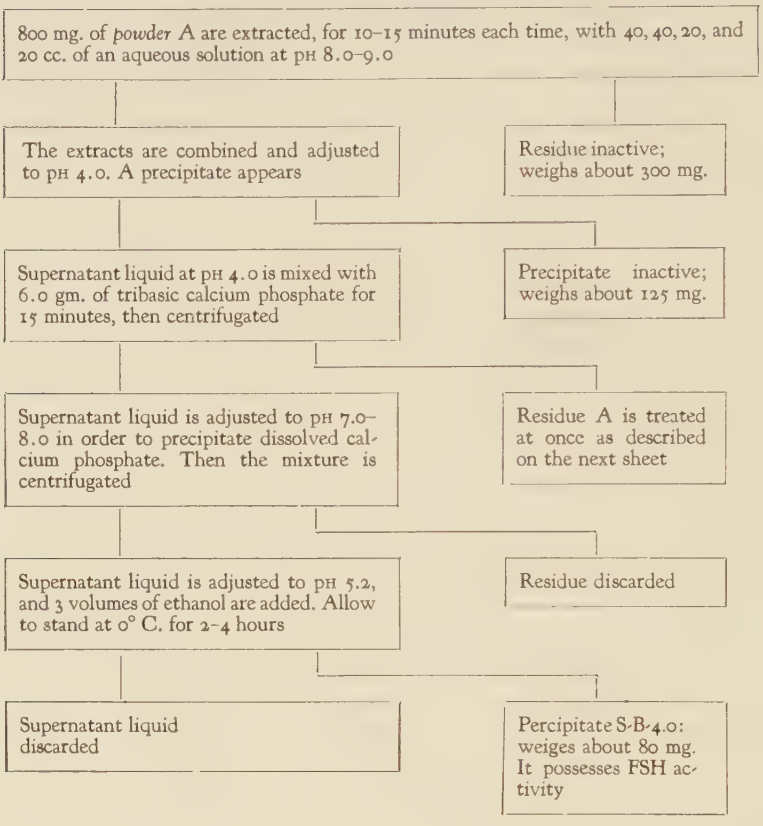
Preparation of Powder EB

Reports in the literature on the separation of the luteinizing (LH) and the follicle-stimulating (FSH) hormones are limited to the use of proteolytic enzymes (12, 13) and to fractional precipitation by alcohol and salts, especially ammonium sulphate (11, 14, 15, 16). No study involving the use of adsorbents seems to have been reported. In this paper are presented the results obtained with the use of adsorbents.

The starting product for these experiments was *powder A*. Preliminary adsorption studies upon aqueous extracts of *powder A* with tribasic calcium phosphate (Merck), Lloyd's reagent (a form of hydrated aluminum silicate), alumina III (aluminum oxide heated to 150°C. for 2 hours), permutit (an artificially prepared aluminum silicate, activated with dilute acetic acid), supercel (essentially silicon dioxide), aluminum hydroxide cream (a colloidal form of aluminum hydroxide used by Hektoen and Welker (17) in their antigen studies), and magnesium oxide, indicated that the last three were useless for concentrating the follicle-stimulating activity under the conditions of our studies. The other four substances all showed definite promise of being of value, but tribasic calcium phosphate (Merck) proved most effective. The details of the ex-

perimental procedure finally adopted, and the yields of the various fractions obtained, are shown on flow sheets 1 and 2.

FLOW SHEET 1. OUTLINE OF THE ADSORPTION PROCEDURE

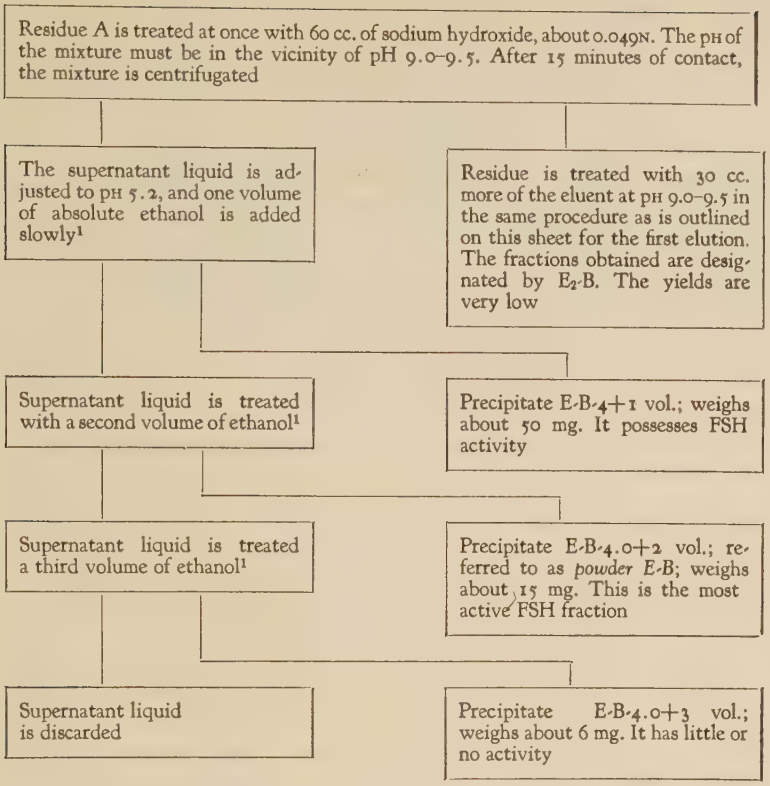


The optimum pH for eluting the follicle-stimulating activity was in the vicinity of pH 9.0 to 9.5. If the pH was maintained above 10.0 during the elution, some destruction of follicle-stimulating activity occurred. If the pH exceeded 11.0, the activity was entirely destroyed, and upon acidification, prior to the addition of alcohol, an odor, similar to that of hydrogen sulphide could be detected. If, during elution, the pH remained below 8.5, the activity was only slowly and partially removed. If the pH was maintained below 7.5, no activity was eluted. A weakly alkaline solution (0.049N sodium hydroxide), adjusted, during the course of elution, to pH 9.0 to 9.5, was as effective as a glycine-sodium hydroxide buffer in eluting the follicle-stimulating activity.

When a 0.02N ammonium hydroxide extract of desiccated sheep pituitary powder was used in place of the aqueous extract of *powder A* as shown in flow sheet 1, the activity and yields of the various fractions were low. This indicates that in the precipitation of *powder A* by alcohol from a 0.02N ammonium hydroxide extract of desiccated sheep pituitary powder (pH adjusted to 5.0 to 5.4 before alcohol addition), some interfering substances are removed.

A number of attempts were made to use a column of calcium phosphate for the adsorption of the follicle-stimulating activity. However, the procedures were too slow and led to very irregular results.

FLOW SHEET 2



[1] The mixture is allowed to stand at 0° C. for a minimum of several hours to facilitate the precipitation.

In several experiments, extracts of *powder A* and solutions of *powder EB* were fractionated with ammonium sulphate by the McMeekin procedure (18) as reported by Fevold et al. (11) except that in some of the experiments, a capillary tube was substituted for the cellophane membrane as the means of introducing the salt. This modification was made in order to lessen the time needed to introduce the ammonium sulphate.

EXPERIMENTAL RESULTS

The gonadotropic activities of the original product and the various actions secured according to flow sheets 1 and 2, and by ammonium sulphate precipitation, are indicated in table 4. These results show that on the addition of the second volume of ethanol to the eluted material, the most potent follicle-stimulating fraction was obtained. This fraction, referred to as *powder EB*, was also assayed in hypophysectomized male and female rats (tables 1 and 2), and the results of those assays have already been discussed.

From the data presented in table 4, it appears that about 50 to 60 per cent of the total follicle-stimulating activity in *powder A* is recovered in the various fractions obtained by means of the adsorption procedure. About one-third of this recovered FSH activity resides in *powder EB*. The data also indicate that *powder EB* is about 3 to 4 times as active as the most potent fraction obtained from an extract of *powder A* by fractional precipitation with ethanol.

TABLE 4. MOUSE ASSAY RESULTS

Body Weight, gm.	Uterine Weight, mg.	Total Dose Injected, mg.	Substance Assayed
		Reference Substances	
9	6.1	0.012	Precipitate obtained when 1 volume of alcohol was added to an extract of *powder A*; 17% yield
9	15.8	0.025	
8	18.5	0.05	
8	6.1	0.012	Precipitate obtained from an extract of *powder A* upon the addition of a second volume of alcohol; 5% yield
8	8.6	0.025	
8	16.3	0.05	
10	6.1	0.05	*Powder A* extract+3rd volume; 2% yield
8	8.5	0.05	*Powder A*
8	13.6	0.10	
8	18.0	0.20	
8	9.7	0.50	Desiccated sheep pituitary powder
8	16.0	1.00	
8	20.9	2.00	
8	5.1	0.00	Controls
		Fractions Obtained by the Adsorption Procedure	
7	11.4	0.05	S-B-4.0; 10% yield
8	18.8	0.05	E-B-4.0+1 vol.; 6% yield
7	8.7	0.006	E-B-4.0+2 vol.; 1.5–2.0% yield (*Powder EB*)
9	19.8	0.012	
8	24.2	0.025	
8	5.1	0.05	E-B-4.0+3 vol.; <1% yield
7	11.3	0.05	E_2-B-4.0+1 vol.; 4% yield
8	7.5	0.012	E_2-B-4.0+2 vol.; 0.5% yield
7	14.1	0.025	
		Fractions Obtained by the Ammonium Sulphate Method	
10	8.7	0.10	LH fraction obtained at 2.1M ammonium sulphate concentration; 21% yield
8	9.8	0.15	
9	11.7	0.33	
9	17.9	0.07	FSH fraction obtained at 3.1M ammonium sulphate concentration; 15% yield
9	18.9	0.13	
11	23.9	0.27	

Measured by the mouse assays, the recovery of the total follicle-stimulating activity by precipitation with ammonium sulphate was of the same order as by our adsorption method. However, the LH fraction obtained at 2.1M ammonium sulphate concentration appeared to have definite follicle-stimulating activity, and the FSH fraction precipitated at 3.1M ammonium sulphate concentration, although of good follicle-stimulating activity, was far less potent than *powder EB*. Several assays in hypophysectomized rats also indicated that the separation of the luteinizing and follicle-stimulating activities was incomplete by the ammonium sulphate precipitation.

Chemical Tests

An attempt to make a quantitative estimation of glycuronic acid by the naphthoresorcinol reaction led to negative results on *powder A* and *powder EB*.[2]

Qualitative protein reactions were carried out on a 1 per cent suspension of *powder A* and a 0.1 per cent solution of *powder EB* in parallel with a 1 per cent casein suspension used as a control. The Molisch, biuret, xanthoproteic, Millon, Ehrlich Diazo, Vanillin, and reduced sulphur tests were positive. The reduced sulphur test was much more strongly positive with the 1 per cent *powder A* suspension than with the 1 per cent casein. Although the 0.1 per cent solution of *powder EB* gave a positive test for reduced sulphur, it was much less intense than with the 1 per cent *powder A* suspension. This test for reduced sulphur appears significant, for, as stated previously, when too basic an eluting agent was used to remove the follicle-stimulating activity adsorbed on calcium phosphate, the activity was destroyed, and upon subsequent acidification of the mixture, an odor similar to that of hydrogen sulphide could be detected. This supports the view of Fraenkel-Conrat, Simpson, and Evans (19) who reported that treating the FSH or ICSH fraction with cysteine resulted in inactivation in both cases.

SUMMARY

1. The sensitivity and reliability of the immature mouse-uterine-weight response as an assay procedure for the follicle-stimulating hormone was confirmed. It was the most sensitive method tried, being 5 to 10 times more sensitive than the hypophysectomized immature-female-rat test and about 20 times more sensitive than the normal immature-female-rat test.

2. Hypophysectomized immature female rats proved to be the most specific for the assay of the follicle-stimulating principle of the pituitary. Hypophysectomized immature male rats were superior to normal intact male rats for the specific determination of the luteinizing hormone.

3. Adsorption of the follicle-stimulating activity from *powder A* on tricalcium phosphate, elution with dilute sodium hydroxide, and fractional precipitation of the eluted activity with ethanol, yielded a very potent follicle-stimulating fraction, *powder EB*.

4. On the basis of comparative assays in hypophysectomized female rats, *powder EB* was found to be 20 times as potent as *powder A* and 200 times as potent as desiccated sheep pituitary powder in follicle-stimulating activity. *Powder EB* was also found to be more potent as a follicle-stimulating preparation than those obtained by fractionation with ammonium sulphate.

5. At the levels assayed, *powder EB* gave negative adrenotropic and thyrotropic assays, and the presence of any appreciable amount of luteinizing activity was questionable.

6. *Powder EB* as well as *powder A* were found to be free of glycuronic acid, but gave positive responses to the common protein and amino acid tests, especially the reduced sulphur test.

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